THE AMINO ACID REQUIREMENTS OF THE CONFUSED FLOUR BEETLE, TRIBOLIUM CONFUSUM, DUVAL.

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The rapidly accumulating literature on the nutrition of insects contains comparatively few data on amino acid requirements. However, all present evidence seems to indicate that insects require the ten amino acids which are essential for the rat.

Work on the nutrition of *Tribolium confusum* in a chemically well-defined medium, consisting of casein, glucose or starch, cholesterol, a salt mixture and eight to 10 vitamins of the B-complex, has been previously published by several authors (Fraenkel and Blewett, 1943, 1947; Fraenkel and Stern, 1951; Offhaus, 1952). An entirely successful "synthetic" diet for *Tribolium,* on which growth is as good as on the best natural diets, has not yet been reported. It has only very recently been found that carnitine is required for adult development (French and Fraenkel, 1954). The addition of 1% brewers yeast to a synthetic diet invariably leads to an improvement of growth. However, even in the absence of yeast, *Tribolium* grows sufficiently well to determine the effect of amino acid deficiencies. The results of the present study largely confirm and extend work on similar lines by Lemonde and Bernard (1951).

Methods

The basic diets used in this investigation were derived from diets which had been previously used in work with *Tribolium.* However, the fact that amino acid mixtures were used in the place of casein necessitated certain modifications in the diet. It was desirable to reduce the proportion of amino acids to a relatively low level which would still allow for adequate growth. *Tribolium* grows well on a wide range of carbohydrates ranging in concentration from 5 to 80% of the diet. In most of our previous work the protein level used was 50%. In the present study this was reduced to a total of 15% casein or mixtures of amino acids. In almost all our previous work the carbohydrate in the diets had been glucose. However, in the present study corn starch was used as the carbohydrate in all tests. Glucose could not be used because of the Maillard reaction between sugars and amino acids described by Friedman and Kline (1950a, 1950b). All the starch used in the experiments to be described was from the same batch.

The diets consisted of 15 parts casein or amino acid mixture, 85 parts corn starch, one part cholesterol, 2 parts McCollum's salt mixture no. 185 and the following vitamins of the B-complex (expressed as μg. per gram of the dry diet): thiamin 25, riboflavin 12.5, nicotinic acid 50, pyridoxin 12.5, pantothenic acid 25, choline chloride 500, inositol 250, pteroylglutamic acid 2.5 and biotin 0.25. All the ingredients, except the vitamins, were mixed in the dry state. To ensure a good distribution of those ingredients which were present in very small amounts,

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the mixture of amino acids was first ground with an equal amount of starch, the cholesterol and salts were then added and ground together, and the balance of starch finally added and mixed in. The diets also contained $NaHCO_3$ in amounts of approximately ten per cent of the amino acid mixture (to neutralize free acids). The vitamins were then added in solution in the amounts stated above to add 10% water to the dry diet. After mixing the vitamin solution into the diet with a spatula, the diets were left standing for two days in a constant temperature chamber at about 30° C. and 60–70% relative humidity, and then ground by hand in a mortar.

The tests were performed in shell vials, 1 × 2 inches, with one gm. of dry diet per vial, two vials to each diet. Ten first stage larvae were placed in each vial. All the tests were performed in a constant temperature chamber at 29–30° C. and 60–70 per cent relative humidity.

To assess the efficiency of a diet, two criteria were used. The number of surviving larvae and their average weight were determined after a period long enough to allow larvae on the optimal diet in a particular experiment to reach their maximum weight before pupation had started. This period varied somewhat, according to the composition of the diets, but was usually 20 days. On optimal diets, with glucose as the carbohydrate and the addition of yeast, pupation may occur after 15 days; however, since all the diets contained starch and few contained yeast, the period required for full growth was longer. The date of pupation was then recorded for each individual larva. From these results the average time to pupation was calculated for each test. In some cases the pupae were kept until the adult beetles emerged and the newly-formed beetles were examined. In following this procedure it was considered possible that certain amino acid deficiencies might affect larval mortality, growth rate, pupation or emergence in a different way than others. The most significant data were usually derived from the weights of the larvae. Since slow growth always leads to a delay in the onset of pupation, a positive correlation should exist between weights, growth rate and days to pupation. However, in some experiments, the number of pupae was unexpectedly small. The data concerning adult emergence finally proved to be without significance, since after most of the work was completed, it was discovered that the adults of *Tribolium,* which were grown on artificial diets, were not viable or failed to emerge, unless carnitine was added to the larval diets. There was, however, no indication that carnitine was necessary for larval growth and successful pupation (French and Fraenkel, 1954).

Growth and survival of *Tribolium* vary somewhat in diets run at different times. This may be due to slight changes in temperature and humidity, a difference in the viability of different batches of larvae and possibly other factors which are not too well understood. It makes it necessary to include in each experiment the appropriate positive and negative controls, and to make strict comparisons only between diets run at the same time.

Experiments

A. The amino acids mixtures used, and their effect on three species of insects

In the absence of data about the amino acid requirements of *Tribolium* when this study was initiated, it was considered advisable to start the work with mixtures which had proved successful with higher animals. Three were used altogether, two of which were amino acid mixtures used by Rose, Oesterling and Womack (1948) with the white rat. The third was one devised by Almquist and Grau (1944) for chicks. Table I gives the percentage composition of these amino acid mixtures. They were at first tested for their effect on the larvae of three beetles, *Tribolium confusum, Tenebrio molitor* and *Dermestes vulpinus.* The diets for *Tribolium* and *Tenebrio* were identical, except for the addition of 1.5 μg. carnitine per gram of the diet for *Tenebrio. Tenebrio* and *Tribolium* received 15% amino acids whereas *Dermestes,* which is a typical protein feeder, received 30% amino

Table I

Composition of the amino acid mixtures used in studies of the amino acid requirements of Tribolium confusum, Tenebrio molitor, and Dermestes vulpinus

Amino acid	Per cent of the total amino acid mixture		
	Rose *et al.*, 1948 19 amino acids	Rose *et al.*, 1948 10 amino acids	Almquist and Grau, 1944 20 amino acids
DL-alanine	2.54		3.33
L-arginine hydrochloride	3.18	4.20	4.67
DL-aspartic acid	2.54		6.67
L-cystine	1.27		1.33
L-glutamic acid	12.69		16.65
Glycine	.64		6.00
L-histidine hydrochloride	6.03	8.15	2.67
L-hydroxyproline	.63		.67
DL-isoleucine	10.16	13.95	6.67
L-leucine	7.62	10.40	6.67
L-lysine	9.53	13.01	4.67
DL-methionine	5.07	6.97	3.33
DL-norleucine			.67
DL-phenylalanine	7.62	11.40	3.33
L-proline	1.27		6.67
DL-serine	1.27		1.33
DL-threonine	8.89	12.10	10.00
L-tryptophane	2.54	3.49	1.33
L-tyrosine	3.81		6.67
DL-valine	12.70	17.35	6.67
$NaHCO_3$	8.07	11.00	5.00
Total	108.07	111.02	105.00

acids, and no carnitine. The results of these tests are given in Table II . *Dermestes* failed to grow on these diets, and *Tenebrio* grew very poorly. *Tribolium,* however, grew on Rose's 19 amino acid mixture as well as it did on casein. With only the 10 essential amino acids in the diet, growth was somewhat delayed. The Almquist mixture proved very much inferior. The experiments with *Tenebrio* and *Dermestes* were first started with first stage larvae. When these larvae failed to develop on the diets, the tests were repeated with larvae of larger size (*Tenebrio* larvae of about 20 mg. and *Dermestes* larvae of about 10 mg.). It was expected that larger larvae which had originally been grown on an optimal diet might be more robust and more able to survive and overcome any adverse effect of amino acid

TABLE II

Response of three insects to amino acid diets

Amino acid mixture and reference	*Dermestes vulpinus*	*Tenebrio molitor*	*Tribolium confusum*
19 amino acids Rose *et al.*, 1948	−	−	++++
10 amino acids Rose *et al.*, 1948	−	±	+++
20 amino acids Almquist *et al.*, 1944	−	±	++
Casein control diet	++++	++++	++++

++++ is growth equal to that on casein.
− is no growth.

diets. However, the larger larvae also failed to develop. All attempts to grow *Dermestes* and *Tenebrio* on amino acid mixtures have so far failed. The good results obtained with *Tribolium* on Rose's mixtures, however, were a starting point for further experiments.

B. The requirements of Tribolium for individual amino acids

Two of Rose's amino acid mixtures were used, one which contained 19 amino acids and another which contained only the 10 "essential" acids, in the proportion shown in Table I. A series of diets was then devised in which each of the amino acids was left out, one at a time. The results were clear cut. In every single case in which one of the 10 essential acids was omitted, the larvae failed to grow (Tables III and IV). Each of the remaining "non-essential" acids could be omitted from the diet, without noticeable effects (Table IV). However, larvae

TABLE III

*Effect on Tribolium larvae of omitting each amino acid from a diet containing the 10 "essential" amino acids**

Exp. 12—weighed at 15 days			Exp. 14—weighed at 20 days		
Diet	No.	Avg. wt. (mg.)	Diet	No.	Avg. wt. (mg.)
Casein control	18	0.55	Casein control	16	1.80
All 10 amino acids	18	0.40	All 10 amino acids	11	0.80
Without L-arginine	10	0.10	Without L-lysine	1	0.10
Without L-histidine	6	0.10	Without DL-methionine	0	
Without L-isoleucine	9	0.08	Without DL-phenylalanine	1	0.10
Without L-leucine	0	—	Without DL-threonine	0	
Without L-tryptophane	13	0.09	Without DL-valine	0	

* None of the larvae on deficient diets survived to pupate.

always grew faster in the presence of 19 amino acids than of ten, in spite of the fact that the total level of amino acids was the same in both instances (Table IV). Superior growth of rats on a mixture of 19 amino acids, as compared with the 10 essential acids, has previously been reported by Rose *et al.* (1948).

TABLE IV

Tribolium confusum. Effect of omitting each amino acid from a diet containing 19 amino acids (Rose diet XXIII)

Experiment 17

Omission or other variation	20 day larvae		Pupae		Adults	
	No.	Avg. wt. (mg.)	No.	Avg. time (days)	No.	No. normal
Casein	15	0.73	14	33.0	14	1
19 amino acids	20	1.11	20	30.1	16	3
No alanine	19	1.14	16	31.4	12	1
No aspartic acid	17	1.18	17	32.2	16	3
No cystine	19	1.17	18	30.9	18	2
No glutamic acid	19	1.11	15	31.4	11	1
No glycine	18	1.31	17	30.3	10	2
No hydroxyproline	12	1.09	10	30.7	9	0
No proline	18	1.00	16	32.7	13	0
No serine	18	0.84	15	32.0	14	3
No tyrosine	19	1.17	19	33.2	16	1
The 10 essential amino acids	18	0.53	12	41.5	11	1
(Rose diet XXIV) Experiment 16						
Casein	19	.96	18	30.7	15	0
19 amino acids						
Ground by hand, mortar	12	.93	9	34.2	8	0
Ground in ball mill—22 hrs.	17	.8	13	35.5	8	3
No arginine	5	.01	0	*	0	0
No histidine	4	.01	0		0	0
No isoleucine	4	.01	0		0	0
No leucine	3	.03	0	*	0	0
No lysine	3	.01	0	*	0	0
No methionine	9	.01	0	**	0	0
No phenylalanine	4	.01	0	**	0	0
No threonine	3	.01	0		0	0
No tryptophane	2	.01	0		0	0
No valine	7	.01	0	*	0	0

* One small larva at 90 days.
** Two small larvae at 90 days.

At the end of 8 weeks, when it was apparent that growth was not possible in the absence of any of the 10 essential amino acids and when most of the larvae had died, 1% yeast was added to each of the deficient diets and the experiment run again with a fresh lot of first stage *Tribolium* larvae. Growth was very much faster after the addition of yeast, and the effect of amino acid deficiencies was largely obscured. This phenomenon is difficult to understand in view of the fact that the addition of 1% yeast only insignificantly adds to the total amount of certain of the essential amino acids in the diet.

Since a diet which contained 19 amino acids always proved superior to one with only 10 essential amino acids, an attempt was made to evaluate the effects of the non-essential amino acids in the diet. Omitting any single non-essential acid had no effect on the diet (Table IV).

It was then considered possible that the amino acids in the mixtures used in the tests might not have been present in optimal proportions. In fact there was no *a priori* reason for such an assumption to be true. Thus those differences in growth rate, which existed between a 10 and a 19 amino acid mixture, might possibly be due to changes in the total amount of some of the acids present. Accordingly, further

TABLE V

Response of Tribolium to D-amino acids in a medium of 19 amino acids

Substitution in diet	D-form substituted				L-form added*			
	20-day larvae		No. of pupae	Av. time to pupation (days)	20-day larvae		No. of pupae	Av. time to pupation (days)
	No.	Av. wt. (mg.)			No.	Av. wt. (mg.)		
Casein control diet	16	0.90	4	27.0				
Amino acid control diet	19	0.60	12	38.0				
D-arginine	6	0.04	0		11	0.66	10	35.0
D-histidine	9	0.05	0		13	0.40	2	38.0
D-isoleucine	0				16	0.30	2	43.0
D-leucine	0				18	0.3	3	41.0
D-lysine	18	0.67	7	34.5				
D-methionine	18	0.80	2	31.0				
D-phenylalanine	17	0.50	0					
D-threonine	0				14	0.30	6	46.3
D-tryptophane	1	0.05	0		15	0.40	4	38.5
D-valine	0				17	0.30	1	39.0

* The L-forms of the respective amino acids were added and the diets re-infested with larvae.

tests with 19 amino acids were devised in which the amount of each amino acid was doubled in individual tests. The hypothesis was that this procedure might produce two kinds of effects. If the diets had been improved, there would have been an indication that the original mixtures did not contain enough of certain acids for optimal growth. If the diets became worse, there would have been an indication that the basic mixtures already might have contained excessive quantities of certain acids. This experiment did not show any clear-cut changes in the efficiency of the diets. Poorer growth resulted with double amounts of aspartic acid and valine. Somewhat poorer growth also resulted when these amino acids were added to a casein diet. However, these effects were only slight.

In further series of tests the D-forms of the ten essential amino acids were individually substituted for the L- or DL-forms, in a diet consisting of 19 amino acids. The D-forms of arginine, histidine, isoleucine, leucine, threonine, tryptophane and valine were entirely inactive (Table V). It was considered possible that some of them might have been not merely inactive, but actually inhibitory. Consequently the diets with D-acids, on which the larvae had failed to grow, were later supplemented with the respective L-form and new larvae added. The larvae grew somewhat slowly on most of the diets, which might have been due to the age of the diets. The results, however, did not suggest that the D-forms were inhibitory.

In one experiment (Table V) the D-forms of lysine and methionine gave as good larval growth as the L-forms, but pupation was fairly good with D-lysine and very poor with D-methionine. D-phenylalanine also showed good growth, but no pupation occurred. In a repeat of this experiment (Table VI), in which carnitine had been added to the diets, D-lysine proved entirely inactive. D-methionine was as active, and D-phenylalanine almost as active as the respective L-forms. All through this test the larvae pupated well and the adults were normal in the presence

TABLE VI

Response of Tribolium to the D-forms of lysine, methionine and phenylalanine in a medium of 19 amino acids

Substitution in diet	20-day old larvae		Pupation		No. of adults	
	No.	Av. weight (mg.)	No.	Av. time (days)	Abnormal	Normal
Casein control diet:						
Carnitine absent	20	.83	20	29.4	17	3
Carnitine present	20	.76	20	29.6	—	20
Amino acid control diet:						
Carnitine absent	16	1.12	12	28.6	12	—
Carnitine present	19	1.68	19	26.1	—	19
D-lysine*	dead	—	—	—	—	—
D-methionine*	17	1.68	17	25.9	—	17
D-phenylalanine*	20	.89	16	29.2	—	16

* Carnitine present.

of carnitine. The result shows that the small number of pupae in the first test might have been, in part, attributable to the absence of carnitine. This, however, would not explain why D-lysine was fairly active in one, and entirely inactive in another test.

Discussion

The results on the amino acid requirements of *Tribolium,* as reported in this paper, closely follow those previously reported for other insects. Lemonde and Bernard (1951), in their work with the same insect, *Tribolium confusum,* reached similar conclusions. They obtained some growth, and even pupation in the absence of either lysine, threonine, phenylalanine, methionine, isoleucine, arginine, leucine and tryptophane, although growth in all these cases was very slow. This may have been due to the presence, in the diets, of one half per cent of yeast. Moore (1946) demonstrated the necessity of the 10 essential amino acids in the nutrition of a carpet beetle, *Attagenus* sp.; the effect of the non-essential acids was, however, not studied. The larva of the yellow fever mosquito, *Aedes aegypti* L. was shown to require glycine for normal growth in addition to the essential amino acids, plus tyrosine for normal pigmentation, and in addition, cystine for normal emergence (Golberg and DeMeillon, 1948). *Drosophila* seems to require, in addition to the 10 essential acids (Schultz *et al.*, 1946; Rudkin and Schultz, 1947) glycine and cystine (Hinton, Noyes and Ellis, 1951). Contrary to some of the aforementioned authors, we have never had any indication that *Tribolium* benefitted by the presence in the diet of cystine or glycine, nor did we find evidence of a toxic effect of Dl- or L-serine as had been reported for *Drosophila* (Hinton *et al.*, 1951).

Information about the nutritional value of the D-form of an essential amino acid has been so far lacking for insects. In the amino acid requirements of man for maintenance of nitrogen equilibrum, D-methionine was as effective as the L-form and D-phenylalanine showed partial activity. The D-forms of valine, leucine, isoleucine, threonine, lysine and tryptophane were inactive (Rose, 1949). In the nutrition of the rat it is generally agreed that the D-forms of tryptophane, phenylalanine and methionine show full or partial activity, while those of the remaining essential acids are inactive (Rose, 1938; Rose *et al.*, 1948; Nasset and Anderson, 1951). For *Tetrahymena* which, in addition to the 10 essential amino acids, also requires serine, the D-forms of methionine, lysine and arginine are active, that of leucine is inhibitory, and those of the remaining six acids are inactive (Elliott *et al.*, 1952). *Tribolium* utilizes fully or partly the D-forms of lysine, methionine and phenylalanine. It therefore appears that the D-methionine is utilized by *Tetrahymena, Tribolium,* the rat and man, D-phenylalanine by *Tribolium*, the rat and man, D-arginine alone by *Tetrahymena,* D-lysine possibly by *Tribolium,* and D-tryptophane alone by the rat.

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Summary

1. The larvae of the flour beetle *Tribolium confusum* have been successfully grown on diets which contain 19 amino acids or the 10 amino acids which are essential in the nutrition of the rat. The larvae of two other beetles, *Tenebrio molitor* and *Dermestes vulpinus,* failed to grow on similar diets.

2. *Tribolium* requires the following amino acids for growth: arginine, histidine, lysine, tryptophane, phenylalanine, methionine, threonine, leucine, isoleucine and valine.

3. On a mixture of 19 amino acids, which in addition to the above-named acids also contains glycine, alanine, proline, hydroxyproline, glutamic acid, aspartic acid, serine, cystine and tyrosine, growth is somewhat faster than in the presence of 10 amino acids.

4. Addition of any one of the non-essential acids to the mixture of the 10 essential ones has no marked effect. None of the amino acids exerted toxic effects when added to the diet in double amounts.

5. *Tribolium* utilizes fully or partly the D-form of methionine, phenylalanine and, possibly, lysine. The D-forms of the 7 remaining essential acids were entirely inactive, but did not show marked toxic effects.

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